The role of local adaptation in shaping fish-mussel coevolution

Running title: Freshwater mussel-host coadaptation

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Abstract

1. The survival of affiliate (dependent) species in a changing environment is determined by the interactions between the affiliate species and their available hosts. However, the patterns of spatial and temporal changes in host compatibility are often unknown despite host shifts having direct impact on the persistence of local populations. Bivalves of the order Unionoida (freshwater mussels) are a functionally important but declining group of affiliate species, which are dependent on freshwater fish to host their parasitic larvae. The role of local adaptations and host fish resistance in shaping freshwater mussel host relationships remains poorly understood.
2. We used an invasive East Asian bivalve, *Sinanodonta woodiana* (Lea 1834), and its potential host fishes to study the mechanisms shaping fish-mussel coevolution using a combination of laboratory cross-exposure methods and field-collected data. We tested whether generalist host use of *S. woodiana* is pertinent to native host species, with special attention to bitterling fishes (Acheilognathinae) that are characterized by a mutual association with freshwater mussels. We also tested whether the pattern of the parasite-host association varies temporally (between areas of ancient and recent sympatry) and spatially (at a sub-basin level in its native range).
3. Results revealed the ability of *S. woodiana* to widely exploit non-bitterling host fishes at a global scale. In contrast, the ability of *S. woodiana* to exploit closely associated bitterling fishes was low in its native range (with ancient sympatry). In areas of recent sympatry (non-native *S. woodiana* range in Europe), *S. woodiana* glochidia were demonstrated to readily parasitize local, evolutionarily naive bitterling species at high density.
4. The results of a population-level experiment with three native populations of *S. woodiana* and rose bitterling, *Rhodeus ocellatus*, from various sub-basins of the River Yangtze confirmed that mussel populations vary in their compatibility with particular host populations. However, there was no evidence of population-specific adaptive coevolution.
5. This study provides the first evidence for a role of fish counter-adaptations against freshwater mussel glochidia, and documents the importance of population-level variation in shaping compatibility between glochidia and their host fishes. This outcome can inform predictions on

the impact of biotic homogenization on endangered affiliate species in general and freshwater mussels in particular.

1 | INTRODUCTION

The existence of many functionally important taxa depends on vulnerable links among interacting species within communities (Koh et al., 2004; Moir et al., 2011). Increasing anthropogenic pressure on freshwater habitats pushes many dependent (affiliate) species to interact with a novel set of potential hosts which may limit their persistence (Vaughn, 2012). A substantial research effort has focused on the consequences of species loss in host-affiliate networks and resulting co-extinction risk (Campião, Ribas, Cornell, Begon, & Tavarez, 2015; Spooner, Xenopoulos, Schneider, & Woolnough, 2011;). This approach has significantly improved our understanding of the risk of imminent impacts, but our knowledge of the potential role of host switching and affiliate-host coevolution remains limited for most affiliate species lineages (Colwell, Dunn, & Harris, 2012).

One of the most characteristic examples of affiliate species in the freshwater environment are freshwater mussels of the order Unionoida (Bivalvia). These benthic invertebrates are a functionally important component of freshwater ecosystems (Vaughn & Hakenkamp, 2001) and their extirpation can lead to long-term losses in mussel-provided ecosystem services (Vaughn, Atkinson, & Julian, 2015). Unionid bivalves are typically dependent on a limited range of species or lineages of freshwater fish (Barnhart, Haag, & Roston, 2008), which are obligatory hosts of their short-term parasitic larval stage (glochidium). Glochidia are not capable of active host selection when released from a parent mussel and die within a few days if they attach to an incompatible host or do not attach at all (Kat, 1984). The lack of compatible hosts in mussel habitats can limit species dispersal (Schwalb, Cottenie, Poos, & Ackerman, 2011), but can also be the critical factor in the decline of many mussel species (Lopes-Lima et al., 2014; Lydeard et al., 2004). While recognition of the importance of host resources for the persistence of mussels is increasing, little is known about the mechanisms of adaptations to host fishes and the evolutionary potential of mussels to adapt to a shifting host community.

In this coevolutionary relationship, the strength of selection on mussels and fish, respectively, is expected to be unbalanced (Barnhart, Haag, & Roston, 2008; Bauer, 1997). Mussels are predicted to experience strong selection on host-compatible genotypes in each generation as the survival of mussel larvae critically depends on host compatibility. In contrast, selection on resistance to glochidia in fish genotypes is likely weaker because mussels are only short-term parasites that usually occupy only a fraction of its host's range and parasitize only a limited proportion of the host population

(Barnhart, Haag, & Roston, 2008; Nuismer, Thompson, & Gomulkiewicz, 2003). As a result, host suitability will be primarily determined by adaptations of mussels to a specific host fish species. However, despite an usually low prevalence and abundance of glochidia in fish populations (Blažek & Gelnar, 2006; Martel & Lauzon-Guay, 2005), recent findings indicate that glochidia can have multiple effects on host fish physiology (Ooue, Terui, Urabe, & Nakamura, 2017; Slavík et al., 2017), behavior (Filipsson et al., 2016; Horký, Douda, Maciak, Závorka, & Slavík, 2014) and condition status (Douda et al., 2016), which can operate at environmentally relevant glochidia densities. These potentially substantial costs to fish of hosting glochidia could play a significant role in mussel-fish coevolution.

An emerging question regards the geometry of local adaptations and host-parasite coevolution between the mussels and fish. Specifically, the coevolution of physiological compatibility may be shaped primarily by mussel adaptations against a non-specific fish immune response, leading to a situation whereby mussels are best adapted to exploit the most available and ecologically compatible host species (*encounter selection hypothesis*). Alternatively, fish may be selected to avoid parasitism by glochidia and evolve resistance to glochidia infection, with the most exposed and ecologically compatible hosts showing greatest resistance. Despite an increase in the understanding of parasite local adaptations (reviewed by Greischar & Koskella, 2007), data on glochidia-fish relationships are limited, and our understanding of host specialisation by freshwater mussels remains opaque.

In this study, we use *Sinanodonta woodiana* (Lea 1834), a unionid mussel with a large natural distribution in Asia (Jing & Zimin, 2013) and invasive elsewhere (e.g. in Europe and Indonesia) (Bolotov et al., 2016; Watters, 1997), using a wide range of potential host fishes. Glochidia of *S. woodiana* are relatively large (390-400 µm), triangular in shape and attach to fish fins and gills where they became encysted, parasitizing the host fish for several days depending on ambient temperature (Dudgeon & Morton, 1984). It has recently been documented that *S. woodiana* can cause significant harm to its hosts (Douda et al., 2016; Slavík et al., 2017), supporting the possibility that potential host species might evolve resistance against *S. woodiana* glochidia. From data collected in the non-native part of its range, *S. woodiana* is considered to be a generalist host whose larvae are capable of development on almost all available fish species (Douda, Vrtílek, Slavík, & Reichard, 2012; Watters, 1997). Data collected in its native range, in contrast, illustrate that there are differences in host quality (Dudgeon & Morton, 1984; Hua, Xu & Wen, 2005). Moreover, data from non-native *S. woodiana* indicate population-specific variability in host compatibility with one host species, the European

bitterling (*Rhodeus amarus*), corresponding with the contrasting histories of recent sympatry between *R. amarus* and *S. woodiana* at the population level (Reichard et al., 2015).

Bitterling fishes are in a unique position with respect to their association with freshwater mussels. Bitterling (Acheilognathinae: Cyprinidae) parasitize freshwater mussels by laying their eggs in the mussel gill cavity where bitterling eggs and embryos complete development over a period of approximately 4 weeks (Smith, Reichard, Jurajda, & Przybylski, 2004). All bitterling, therefore, spend some part of their lives in close association with adult mussels. Notably, the bitterling mating system involves males guarding territories around freshwater mussels where reproductively active females are courted over an extended reproductive period (2-6 months). The spawning behaviour of male and female bitterling involves frequent intimate association with adult mussels, such as regular inspection of mussel siphons (Phillips, Reichard, & Smith, 2017; Spence, Reichard, & Smith, 2013). Such behavior makes bitterling potentially vulnerable to glochidial infection, particularly as mussels expel glochidia during late spring and early summer at the height of the bitterling spawning season.

The aim of this study was to test whether *S. woodiana* functions as a host generalist in both the native and non-native part of its range, using a combination of laboratory experiments and data from natural populations. By comparing infection patterns in the native and non-native range of *S. woodiana* we were able to investigate whether the outcome of parasite-host conflict varied between the areas of ancient sympatry and recently invaded area (long-term temporal axis) and over a spatial scale (at a sub-basin level in the native range). The specific aims of the study were to: 1) experimentally compare the physiological compatibility of *S. woodiana* glochidia for infecting bitterling and non-bitterling fish between a native and non-native range; 2) experimentally evaluate the degree of population-level variation in host compatibility in *S. woodiana*'s native range and relate to the results for its non-native range; 3) quantify the natural levels of *S. woodiana* glochidia infection of fishes in its native and non-native range and thus; 4) evaluate the implications for the evolution of host relationships in freshwater mussels.

2 | METHODS

2.1 | Species level host compatibility of *S. woodiana*

Study populations were located in the River Yangtze Basin (Fig. 1), which is considered to be the likely site of origin of invasive European populations (Bolotov et al., 2016). This is also the area of ancient sympatry between *S. woodiana* and several bitterling species (Reichard, Liu, & Smith, 2007). Gravid females were collected in May and June 2014 from Lake Bao'an (population B, Hubei province, China, 30°17'25.4"N 114°43'48.9"E). Female mussels were transported to the laboratory and stored in 1350 L shallow containers with aerated tap water. Ripeness of mussel glochidia was regularly checked by visual inspection of marsupium colour (a dark purple colour indicates advanced developmental stage, K. Douda, personal observation). Ripe glochidia were obtained by flushing the marsupium with water from a syringe. The viability of glochidia (a subset of 30 individuals for each mussel) was verified by evaluation of their snapping action in a sodium chloride solution. Glochidia from three gravid females with a viability exceeding 90% were pooled and used for inoculation. Nine potential host species (Table 1) from four fish families (Cyprinidae, Siluridae, Gobiidae, Cobitidae) were used for experimental infection by glochidia. *Carassius auratus, Rhinogobius giurinus, Hypophthalmichthys nobilis, Sinibotia superciliaris, Silurus meridionalis, Plagiognathops microlepis* are native Chinese fish species with extensive range overlap with *S. woodiana*. As a control, we also included *Danio rerio*, a species of Indian origin (Spence, Gerlach, Lawrence, & Smith, 2008). Two common and widely distributed Asian bitterling species, *R. ocellatus* and *R. sinensis*, were tested as hosts with a persistently high risk of parasitism. Experimental fish were obtained from commercial hatcheries or rearing facilities of the Institute of Hydrobiology of the Chinese Academy of Science in Wuhan, except for *R. giurinus* and both *Rhodeus* species which were collected using dip nets net from Lake Bao'an. Seven to twenty-six individual fish per species were used for infections; a total of 105 individually monitored fish (Table 1). Experimental fish were either in the first or second year of life. Six fish (four *H. nobilis* and two *C. auratus*) died before the end of juvenile detachment (mortality was not related to experimental infection) and were not included in the analysis.

Fish were infected following the protocol of Douda, Vrtílek, Slavík and Reichard (2012) by placement into a common bath suspension (one infection tank for all species, total volume 50 L) of dechlorinated tap water and containing 3860 ± 2097 (mean ± s.d.) viable glochidia L^{-1}. The glochidia were kept in homogeneous suspension through aeration. After 15 min of inoculation, fish were transferred into an aerated bath that did not contain glochidia for 30 min to rinse off non-attached glochidia. Fish were

then individually placed into 105 continuously aerated 5-L plastic tanks with dechlorinated tap water that had nets with 3 mm mesh size on the bottom. Fish were fed daily with a commercial flake fish food. The temperature in the tanks was 24.9 ± 0.5 °C (mean ± s.d.) during the experiment. Water in individual tanks was filtered through nylon screens (mesh size 139 µm; UHELON) and partially exchanged (80% of water volume) every second day for 14 days in total. Untransformed glochidia and juvenile mussels were washed from the screens, isolated from debris and counted under a stereomicroscope. All individuals that were collected were inspected; if foot activity or valve movement were observed, individuals were classified as live juveniles.

In order to compare experimental data on physiological host compatibility between the native and non-native range of *S. woodiana* we used the results of host compatibility testing by Douda, Vrtílek, Slavík and Reichard (2012) as a comparative dataset. Data on the metamorphosis success rate of *S. woodiana* and eight common fish species in Central Europe (family Cyprinidae, Supl. Table S1), including one bitterling species (*Rhodeus amarus*), were compared with experimental data from the native range of *S. woodiana* collected in the present study. The European dataset included metamorphosis success from six to ten individuals per fish species (age 0+, 1+) infested experimentally with *S. woodiana* glochidia from the River Kyjovka (Danube basin) where the mussel has been introduced (see Douda, Vrtílek, Slavík, & Reichard, 2012 for further details).

2.2 | Population level host compatibility of *S. woodiana*

Gravid females of *S. woodiana* were collected in May - June 2014 from three different populations in the River Yangtze Basin, China (Fig. 1). Mussels syntopic with the experimental *R. ocellatus* population were collected from Lake Bao'an (population B). Mussels syntopic with their local (non-experimental) *R. ocellatus* population were collected from earthen fishponds near Jianli (population A, Hubei province, 29°58'37.5"N, 112°42'29.4"E). Mussels naïve to *R. ocellatus* were collected from Lake Qinglan (population C, Jiangxi province, 28°30'40.144"N, 116°8'2.112"E). The study populations of *S. woodiana* were 187-353 km apart and were located in separated watersheds of three distinct tributaries of the River Yangtze.

The majority of host fishes used in the study (syntopic *R. ocellatus, R. sinensis, R. giurinus*) were collected at one of the mussel collection sites (population B, Bao'an Lake) in April and May 2014 using baited fish traps. The second population of *R. ocellatus* (naïve to experimental populations of mussels) was collected in a natural pond in the city of Nanchang (Jiangxi province, 28°39'55.08"N, 115°48'57.79"E), located 35 km from Lake Qinglan (mussel population C collection site). All fish were transported to the laboratory in aerated boxes at least three weeks before the experiments to ensure that no glochidia were attached to experimental fish. Artificially reared *Gobiocypris rarus* Ye & Fu, 1983, a cyprinid fish species from the native range of *S. woodiana* with an endemic and highly restricted distribution, was obtained from a local rearing facility (Institute of Hydrobiology, Chinese Academy of Science) and included in the analysis as a control species. The mean ± s.d. standard length and mean ± s.d. wet body mass of experimental fish are reported in Table S2.

We used larvae from one parent mussel to simultaneously infect two to four individuals from each tested fish strain (*R. ocellatus* – Lake Bao'an, *R. ocellatus* – Lake Qinglan, *R. sinensis*, *G. rarus*, *R. giurinus*). Altogether, we performed 23 experimental infections (7-8 female mussels per each of the three tested populations) to infect a total of 220 individual fish (14–16 individuals for each fish strain–*S. woodiana* population combination). The fish were infected in aerated suspensions (0.5 L per fish) with mean larval densities of 2885 ± 641 (mean ± s.d.) viable glochidia L^{-1}. A common infection bath suspension was used in each inoculation, which was subdivided into five sections to prevent mixing of fish individuals from different source populations. After a 15-min exposure, fish were transferred to clean water for 30 min to rinse non-attached glochidia.

Fish were subsequently placed singly into continuously aerated 5 L plastic tanks (i.e. a total of 220 tanks). An initial host response was monitored using the same methods as for the species-level experiment. Rejected larvae were collected from water using filters and counted 48 hours after infection. The number of total attached glochidia was estimated by host dissection and direct enumeration of remaining glochidia on the body of each fish. Mean water temperature was 25.0 ± 0.8 °C (mean ± s.d.) during the experiment. These methods enabled us to estimate both the absolute number of attached larvae and the proportion of larvae that successfully survived the initial host response. No fish died during the experiment.

We used the ratio between the number of initially attached glochidia per fish and the proportion of glochidia that remained attached 48 hours after infection as a measure of glochidia–fish physiological compatibility, hereafter referred to as 'infection success'. This parameter was used because the initial host response represents a critical stage in the parasitic phase of unionid bivalve larvae (Dodd, Barnhart, Rogers-Lowery, Fobian, & Dimock, 2005; Rogers-Lowery & Dimock, 2006). Additionally, it has been shown that the shedding of immature glochidia in the initial period after infestation closely corresponds to the overall success of glochidia parasitism (Douda, Horký, & Bílý, 2012). The absolute number of attached glochidia per gram of fish body weight was also calculated for the experimental fish and compared among treatments in order to compare relative susceptibility of fish strains to glochidia attachment.

2.3 | Natural prevalence and abundance of *S. woodiana* glochidia on host fish

In order to complement physiological data with an understanding of ecological host compatibility in *S. woodiana* we also quantified natural encystment rates. We quantified the prevalence and intensity of infection of *S. woodiana* glochidia on fish collected in the field during the glochidia discharge season in both the native Asian (Lake Bao'an) and non-native European range (River Kyjovka). Sites with the occurrence of bitterling species were selected to enable the comparison of relative use of bitterling species as hosts.

Fish were collected from the River Kyjovka using a standard electrofishing method (pulsed DC, 50 Hz, Lena, Czech Republic) between July 10th 2013 and August 22nd 2015. Electrofishing has no impact on glochidial attachment (Holliman, Kwak, Cope, & Levine, 2007). The River Kyjovka (Czech Republic) is a small lowland river (4-6 m wide, <1 m deep) typical of the region. In Lake Bao'an, fish were collected between May 23rd and June 12th 2014 using baited fish traps, which was the most effective method for collecting small-sized fish at the site.

At both sites sampled fish were killed with an overdose of aesthetic (MS-222) and immediately placed in a fixative (70% ethanol, 4% formaldehyde) and transported to the laboratory. Glochidia of *S. woodiana* were identified on the basis of characteristic morphology and counted under a dissection stereomicroscope.

2.4 | Statistical analysis

Glochidia metamorphosis success on different host species (proportion of glochidia that successfully transformed into juvenile stage) was compared among host species using generalized linear models (GLM, logit link function and a quasi-binomial error structure). The dependent variable (metamorphosis success) was treated as binary. Multiple comparisons were performed by Tukey's post-hoc test using the *glht* function in the R package *multcomp* (Hothorn, Bretz, & Westfall, 2008) to examine pairwise differences among host species.

Data on infection success of glochidia (proportion of glochidia that remained attached after 48 hours) on different fish strain by population combinations were analysed in a generalized linear mixed model framework (GLMM, logit link function and quasi-binomial error structure, *glmer* procedure), and data on initially attached glochidia per gram in a linear mixed model framework (LMM, *lmer* procedure) in the R package *lme4* (Bates, Maechler, Bolker, & Walker, 2015). The dependent variable (infection success) was treated as a binary variable. The fixed effects included *Mussel population* (populations A - Jianli, B - Bao'an, C - Qinglan), *Fish strain* (sympatric *R. ocellatus,* allopatric *R. ocellatus, R. sinensis, R. giurinus, G. rarus*) and their interaction *Mussel population x Fish strain*. Random effects (intercepts) included *Mussel individual* (infestation event) and *Host fish individual* (not included in the model on attached glochidia per gram).

The comparison of *S. woodiana* glochidia natural infestation rates (number of recovered glochidia) on bitterling and non-bitterling fish between native and non-native range sites was performed using Fisher's Exact Test for Count Data (Sokal & Rohlf, 1995). All analyses were performed using the R 3.3.2 software package (R Core Development Team, 2016).

3 | RESULTS

3.1 | *Sinanodonta woodiana* host compatibility at the species level

Glochidia metamorphosis success rate differed significantly among host fish species (Fig. 2; GLM: $F_{10,142} = 17.0$, $p < 0.001$). Mean metamorphosis success rate of *S. woodiana* on Asian non-bitterling

host species was high and ranged from 57.6 to 87.5% (Table 1), implicating that all Asian non-bitterling fish included in the study were physiologically suitable hosts for *S. woodiana*. In contrast, most attached glochidia were shed from both bitterling fish species tested in the first few days post infection, resulting in low metamorphosis success rates of 1.4 - 3.5% (Table 1). Comparing metamorphosis success rate data between native and non-native ranges revealed that an intermediate level of metamorphosis success rate was recorded in Europe for both the bitterling (metamorphosis success 21.7 ± 13.1%) and non-bitterling species (metamorphosis success 43.4 ± 15.2%) (mean ± s.d.). The statistical significance of pairwise comparisons is shown in Fig. 2.

3.2 | *Sinanodonta woodiana* host compatibility at population level

The number of initially attached *S. woodiana* per gram of fish body weight (recorded at the beginning of the monitoring period, 30 minutes after infection) differed due to variation among host fish strains (χ^2 = 561.3, p < 0.001) but not between *S. woodiana* populations (Table 2; χ^2 = 3.78, p = 0.150). Bitterling strains had considerably lower numbers of attached glochidia 30 minutes after infection compared to the control fish species (Fig. 3). There was also a significant interaction between the source mussel population and fish strain (χ^2 = 38.3, p < 0.001) and a significant effect of the source mussel individual (χ^2 = 15.4, p < 0.001).

The infection success of *S. woodiana* demonstrated a similar pattern to the initial attachment rate. It differed due to the variation among host fish strains (GLMM: χ^2 = 296.6, p < 0.001) but not between *S. woodiana* populations (Table 2; χ^2 = 3.36, p = 0.190). The results corroborated the outcome of the species-level experiment; the control fish species *G. rarus* and *R. giurinus* had considerably higher infection rates by *S. woodiana* than all bitterling strains tested (Fig. 3). Although the three *S. woodiana* populations did not differ in their capacity to infect their hosts, there was a significant interaction between source mussel population and fish strain (χ^2 = 28.4, p < 0.001). A significant effect of random factors indicated an additional role of individual-level variability among individual fish (χ^2 = 463.14, p < 0.001) and infections from a specific source of mussels (χ^2 = 7.33, p < 0.01). There were no indications of elevated absolute attachment rate or higher infection success in the sympatric mussel-fish combinations (i.e. fish strains and mussel population from Lake Bao'an) (Fig. 3A,B).

3.3 | Natural prevalence of *S. woodiana* glochidia on host fish

In total, 123 fish belonging to 10 species (including 6 bitterling species) were captured and dissected in the native range site (Lake Bao'an) of *S. woodiana* and 243 fish from 10 species (including 1 bitterling species) in its non-native range (River Kyjovka). Glochidia were attached to the fins, gills and other body parts (mouths, opercula) of fish. Summary results of glochidia quantification are presented in Table 3. The highest glochidia prevalence per species reached 24% for *R. giurinus* and 78% for *C. carpio* at Lake Bao'an and River Kyjovka respectively. The maximum number of *S. woodiana* glochidia per individual fish was 45 larvae recovered from *P. parva* (fish body weight 7.7 g) from the River Kyjovka.

In the native range the proportion of bitterling fish parasitized by *S. woodiana* was significantly lower than the proportion of parasitized non-bitterling fish species (Fisher's Exact Test for Count Data: n = 123, $p = 0.003$). In contrast, there were no differences between the proportion of parasitized bitterling and non-bitterling fishes in the non-native European range (n = 243, $p = 0.570$).

4 | DISCUSSION

4.1 | The role of fish counter adaptations against glochidia

This study identifies clear differences in the patterns of bitterling fish utilization by *S. woodiana* glochidia between populations in its native and non-native range. The capacity of *S. woodiana* to exploit non-bitterling fish is relatively high in both areas indicating low dependence on coevolutionary history with specific host taxa. Using the example of European unionids, Bauer (2001) proposed that host specificity of freshwater mussels is primarily determined by available host range. Mussel species that occur in specific habitats with a relatively homogenous fish fauna tend to be host specialists, while species from more diverse and species-rich habitats are host generalists (Bauer, 2001). Thus, a host generalist strategy is supposed to be advantageous in less predictable environments with a diverse and variable fish community, where the survival of glochidia is increased by their ability to utilize a wide range of fish species. At the same time, host generalism may be costly as generalist parasites

are often less capable of exploit their hosts efficiently in comparison with specialist parasites (Leggett, Buckling, Long, & Boots, 2013). Earlier studies document that the glochidia of host generalist freshwater mussels are produced in lower numbers than host specialists, though they are larger and at a more advanced stage of development. Notably, they do not grow on their host fish and detach earlier than glochidia of specialists, possibly before the host specific immune response can be initiated (Bauer, 2001). From this perspective, *S. woodiana* expresses life-history traits predicted for a high level of host generalism (Douda, Vrtílek, Slavík, & Reichard, 2012; Dudgeon & Morton, 1984; Hua, Xu, & Wen, 2005) and conclusion supported in the present study based on non-bitterling host species.

While Bauer‘s (2001) concept provides insights into the selective forces driving host range evolution in freshwater mussels, Haag (2012) demonstrated several notable exceptions from the general pattern of generalist-specialist continuum in the diverse North American mussel fauna. Similarly, the present study demonstrated that host generalism in *S. woodiana* has its limitations. Contrasting the two sets of data – experimental exposure (Table 1, Fig. 3) and field-collected data (Table 3) between the native and invasive range (Figure 2, Table 3) revealed that evolutionarily sympatric bitterling species, which face an unusually high risk of parasitism by *S. woodiana*, were inferior hosts for *S. woodiana* glochidia in its native range (China). In contrast, an evolutionarily naïve bitterling species (*R. amarus*) was readily parasitized by *S. woodiana* in its non-native range (Europe). This finding indicates that *S. woodiana* can temporarily capitalize on the coevolutionary naivety of hosts during range expansion. Interestingly, significant temporal shifts in host quality have already been recorded after a few generations (Reichard et al., 2015), with European bitterling sympatric with *S. woodiana* for little more than a decade in European waters becoming less suitable hosts for *S. woodiana*.

Results from this study do not support the hypothesis that the outcome of the mussel-fish relationship is exclusively determined by mussel adaptations without fish counter-adaptations. The glochidia of *S. woodiana* were able to overcome the immune system of almost all hosts tested, including completely allopatric species. However, our data also indicate that if the risk of parasitism is consistently high (such as for the bitterling fishes in *S. woodiana*'s native range), fish are capable of evolving efficient resistance to glochidia. The lack of compatibility with host species that experience high encounter rates with *S. woodiana* glochidia in their natural habitat contradicts predictions from the encounter selection hypothesis.

Both innate and acquired host immunity was identified by previous studies to be involved in the protection of fish against glochidia of freshwater mussels (Bauer, 2001; Dodd, Barnhart, Rogers-Lowery, Fobian, & Dimock, 2006; Rogers-Lowery, Dimock, & Kuhn 2007), including *S. woodiana* (Donrovich et al. 2017). In the present study, we were not able to separate the roles of innate and acquired immunity in bitterling fish resistance because we used wild-caught individuals for which we cannot exclude potential previous contact with glochidia. Nevertheless, the high metamorphosis success rate of *S. woodiana* glochidia on control fish species collected from the same sampling site (*R. giurinus*) together with low glochidia prevalence on the host fishes in that collection site (Table 3) indicate a relatively minor role of potential previous contact with glochidia. This assumption is supported by the fact that in a comparable study of host compatibility of European bitterling and *S. woodiana*, field collected fish were also used and the study showed high metamorphosis success rates of glochidia (Reichard et al., 2015). Also, natural infection levels of *S. woodiana* on bitterling and non-bitterling fish in both study areas are congruent with the experimental data. However, a different experimental approach will be needed to compare the roles of innate and acquired immunity in bitterling resistance to mussel parasitism.

The benefits of fish immunological resistance to glochidia can be relatively small because hosting these short-term parasites can incur a comparable cost to sloughing them (Barnhart, Haag, & Roston 2008). Our finding that evolutionarily sympatric bitterling fishes are protected against *S. woodiana* glochidia indicates that such resistance can be cost-effective and that the fitness cost imposed by parasitism is higher than the cost of the response. An alternative explanation is that *S. woodiana* glochidia actively prefer other fish taxa as hosts, with apparent resistance of sympatric bitterling an artefact. However, this explanation is unlikely given the large range of hosts used in our experiment and in previous studies (Dudgeon & Morton, 1984; Hua, Xu, & Wen, 2005; Reichard et al., 2015). Effective resistance by bitterling to glochidia is striking in the context of the potentially mutualistic relationship between bitterling and freshwater mussels. Supporting successful development of glochidia will lead to the establishment of new spawning sites for bitterling. The hypothesis of a mutualistic relationship between the bitterling and mussels has been repeatedly questioned (reviewed in Smith, Reichard, Jurajda, & Przybylski, 2004). Our results corroborate earlier rejection of the hypothesis (e.g. Reichard, Ondračková, Przybylski, Liu, & Smith, 2006; Spence & Smith, 2013). Thus, low prevalence of glochidia on bitterling fish implies a negligible benefit to individual bitterling in acting

as mussel hosts. How bitterling avoid glochidial infection, either through behavioural or morphological adaptations, or whether they lose glochidia after a brief initial attachment through an immune response (Smith, Reichard, Jurajda, & Przybylski, 2004), is not known. Whatever the mechanism, based on the present study and the results of Reichard et al. (2015), it is apparent that the coevolutionary relationship between freshwater mussels and bitterling fishes is driven by an antagonistic arms races rather than a mutualistic coevolutionary equilibrium.

Overall, our data indicate that fish can evolve a physiological resistance to the glochidia of a highly infective generalist mussel with a short-term parasitic phase. It can be hypothesized that similar resistance can occur in other mussel-fish species combinations. More detailed studies are needed to understand the evolution of host utilization by unionid bivalves, as both mussel adaptation and specific host resistance are likely to be involved in mussel-fish physiological compatibility.

4.2 | The role of local adaptations in mussel-fish relationships

The population scale experiment presented in this study focused on differences in host compatibility between three native range populations of *S. woodiana* inhabiting discrete River Yangtze sub-basins in its native range. The results of this common-garden experiment are in agreement with the previous findings indicating that different mussel populations can vary in their compatibility with particular host strains/populations (Douda et al., 2014; Eckert, 2003; Engel & Wachtler, 1989; Karlsson, Larsen, & Hindar, 2013; Reichard et al., 2015; Rogers, Watson, & Neves, 2001; Serb & Barnhart, 2008). Our finding is unique in documenting population-level variation in host compatibility even for a host generalist taxon within a single river system. This finding adds support for the importance of host-affiliate studies in species management unit recognition (Douda et al., 2014).

We hypothesized that the bitterling-glochidia relationship may be population-specific. However, we did not detect significant differences in the ability of *S. woodiana* glochidia to infect sympatric and allopatric populations of *R. ocellatus*, as predicted under the encounter selection hypothesis (Eckert, 2003; Rogers, Watson, & Neves, 2001). There was also no support for adaptive differences in host compatibility at the population scale in our study system. These results, together with other recently published evidence (Caldwell, Zanatta, & Woolnough, 2016; Douda et al., 2014; Österling & Larsen,

2013; Reichard et al., 2015) indicate that specific local adaptations (i.e. between coexisting populations) of mussels and fish are not as common in contemporary communities as previously suggested. However, the lack of evidence for specific local adaptations does not imply that these patterns do not evolve in less dynamic systems with restricted gene flow in mussel and fish populations, or that they have not been common in the past. It is possible that many local adaptations have been eroded through human influence on freshwater biota. *Sinanodonta woodiana* is an economically important species, used as a food source and for other purposes in its native range (Chen, Liu, Su, & Yang, 2015). Transfer of mussels within their native range are common in south-east Asia (Douda, personal observation) and the situation can be even more pronounced for its host fishes.

In conclusion, our study contributes to the understanding of the potential role of local adaptations and host resistance in shaping freshwater mussel-fish relationships. The inter-continental spread of *S. woodiana*, a host generalist, provides evidence for the role of fish counter-adaptations against glochidia infection, documented in laboratory experiments and directly quantified under natural conditions. The current pace of biotic homogenization of freshwater ecosystems forces many species of freshwater mussel and fish to interact with novel partners (Douda et al., 2013; Poos, Dextrase, Salonen, Marjomäki, & Taskinen, 2016; Schwalb, & Ackerman, 2010; Šlapanský, Jurajda, & Janáč, 2016; Tremblay, Morris, & Ackerman, 2016) and local adaptations and counter-adaptations may play a significant role in the quality of host resources available for particular mussel species. Given the global decline of freshwater mussels (Lopes-Lima et al., 2014), including native *S. woodiana* populations in China (Chen et al., 2015), further studies are needed to investigate the impact of host limitation. Unionid bivalves represent functionally important components of freshwater systems and the vulnerability of their host-affiliate relationships will determine the future role of these species in changing freshwater ecosystems. A better understanding of how natural selection drives the evolution of mussel infectivity and fish resistance will be crucial for predicting the impact of biotic homogenization on freshwater mussels. Ultimately, it may lead to better protection of host resources for this endangered taxon.

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TABLE 1. Results of *Sinanodonta woodiana* host compatibility test at the species level. The number of host fish individuals (N_H), mean (± s.d.) values of fish body weight (W), standard length (SL), the number of glochidia attached (N_G), and metamorphosis success (M) are shown. Attached glochidia represent the sum of glochidia and metamorphosed juveniles recovered from individual fish; metamorphosis success indicates % of attached glochidia that were recovered as live juveniles. Mean values denoted by the same superscript letter are not significantly different (Tukey's HSD test at $p < 0.05$). Bitterling hosts highlighted in bold.

Species	N_H	W (g)	SL (mm)	N_G	M (%)
***Rhodeus ocellatus* (Kner, 1866)**	**26**	**0.83 ± 0.36**	**34.3 ± 3.9**	**57.7 ± 59.0**	**1.4 ± 3.7 ^{C}**
***Rhodeus sinensis* Günther, 1868**	**11**	**1.18 ± 0.48**	**37.7 ± 4.5**	**87.8 ± 61.1**	**3.5 ± 4.2 ^{C}**
Carassius auratus (Linnaeus, 1758)	8	0.37 ± 0.20	24.7 ± 4.4	55.5 ± 15.0	85.4 ± 3.8 ^{A}
Danio rerio (Hamilton, 1822)	10	0.37 ± 0.04	27.8 ± 0.5	63.2 ± 18.7	65.0 ± 15.4 ^{AB}
Rhinogobius giurinus (Rutter, 1897)	11	0.94 ± 0.20	41.2 ± 2.5	175.1 ± 73.6	60.0 ± 21.2 ^{B}
Hypophthalmichthys nobilis (Richardson, 1845)	6	0.20 ± 0.06	21.7 ± 3.6	21.8 ± 13.4	87.5 ± 6.9 ^{AB}
Sinibotia superciliaris (Günther, 1892)	10	1.01 ± 0.24	41.6 ± 3.2	96.3 ± 22.5	57.6 ± 16.0 ^{B}
Silurus meridionalis Chen, 1977	7	1.42 ± 0.88	55.4 ± 12.1	272.0 ± 114.5	57.9 ± 9.9 ^{B}
Plagiognathops microlepis (Bleeker, 1871)	10	0.13 ± 0.03	20.7 ± 2.0	18.8 ± 5.7	75.2 ± 13.6 ^{AB}

TABLE 2. Sources of variation in host compatibility between *Sinanodonta woodiana* and its native-range hosts. Effects of multiple variables on the number of attached glochidia per gram of fish body weight (using *lmer* in R) and infection success (using *glmer* in R). The results show the effects of fixed factors *Mussel population* (populations A - Jianli, B - Bao'an, C - Qinglan), *Fish strain* (sympatric *R. ocellatus,* allopatric *R. ocellatus, R. sinensis, R. giurinus, G. rarus*) and their interaction, as well as random effects of *Mussel individual* and *Host fish individual*. Chi-square values, degrees of freedom (DF), and *P*-values are shown for the likelihood-ratio tests between full models and models with each factor removed. The effects that improved the model fit significantly ($p < 0.05$) are highlighted in bold.

	A. Attached per gram		**B. Infection success**	
	X^2 (DF)	*p*-value	X^2 (DF)	*p*-value
Fixed Factors				
Mussel pop.	3.78 (2)	0.15	3.36 (2)	0.19
Fish strain	561.3 (4)	**<0.001**	296.6 (4)	**<0.001**
Mussel pop. x Fish strain	38.3 (8)	**<0.001**	28.4 (8)	**<0.001**
Random Factors				
Mussel ind.	15.4 (1)	**<0.001**	7.3 (1)	**<0.01**
Host fish ind.	-	-	463.1 (1)	**<0.001**

TABLE 3 The natural prevalence and intensity of infection of *Sinanodonta woodiana* glochidia on fish collected from the field during the glochidia discharge season at study populations from its native (Lake Bao'an; China) and non-native range (River Kyjovka; Czech Republic). The number of host individuals (N_H), mean (± s.d.) values of fish body weight (W), standard length (SL), the number of infected hosts (N_{INF}), maximum number of glochidia on individual fish (Max), mean intensity of infection (Intens) and glochidia prevalence (Prev) are shown. Prevalence indicates the proportion of fish infected. Mean intensity of infection indicates the average number of *S. woodiana* glochidia per infected host. Bitterling hosts highlighted in bold.

	Fish species	N_H	W (g)	SL (mm)	N_{INF}	Max	Intens	Prev
Native range	***Acheilognathus barbatulus* Günther, 1873**	**35**	**2.17 ± 0.86**	**45.7 ± 6.2**	**2**	**2**	**1.00**	**0.06**
	***Acheilognathus macropterus* (Bleeker, 1871)**	**1**	**3.55**	**57**	**0**	**0**	**-**	**0.00**
	***Acheilognathus tonkinensis* (Vaillant, 1892)**	**3**	**2.62 ± 0.59**	**49.0 ± 5.0**	**0**	**0**	**-**	**0.00**
	***Paracheilognathus imberbis* (Günther, 1868)**	**6**	**1.22 ± 0.46**	**39.8 ± 5.6**	**0**	**0**	**-**	**0.00**
	***Rhodeus sinensis* Günther, 1868**	**2**	**0.87, 0.68**	**32, 29**	**0**	**0**	**-**	**0.00**
	***Rhodeus ocellatus* (Kner, 1866)**	**33**	**0.99 ± 0.33**	**33.2 ± 4.2**	**0**	**0**	**-**	**0.00**
	Carassius auratus (Linnaeus, 1758)	12	0.80 ± 0.32	31.4 ± 4.5	2	3	1.50	0.17
	Hemiculter leucisculus (Basilewsky, 1855)	2	2.54, 2.92	69, 73	0	0	-	0.00
	Mastacembelus aculeatus (Bloch, 1786)	8	4.56 ± 3.08	120.1 ± 13.2	1	1	1.00	0.13
	Rhinogobius giurinus (Rutter, 1897)	21	0.82 ± 0.19	34.9 ± 3.1	5	6	1.20	0.24
Introduced range	***Rhodeus amarus***	**96**	**0.45 ± 0.79**	**19.6 ± 10.1**	**26**	**45**	**1.73**	**0.27**
	Blicca bjoerkna (Linnaeus, 1758)	23	7.45 ± 3.90	64.2 ± 11.3	3	3	1.00	0.13
	Alburnus alburnus (Linnaeus, 1758)	25	4.75 ± 2.58	65.9 ± 10.3	0	0	-	0.00
	Scardinius erythrophthalmus (Linnaeus, 1758)	23	6.52 ± 3.38	62.8 ± 11.0	11	48	4.36	0.48
	Rutilus rutilus (Linnaeus, 1758)	11	6.88 ± 3.62	65.6 ± 16.4	0	0	-	0.00
	Pseudorasbora parva (Temminck &	31	2.70 ± 2.26	46.5 ± 14.8	14	127	9.07	0.45

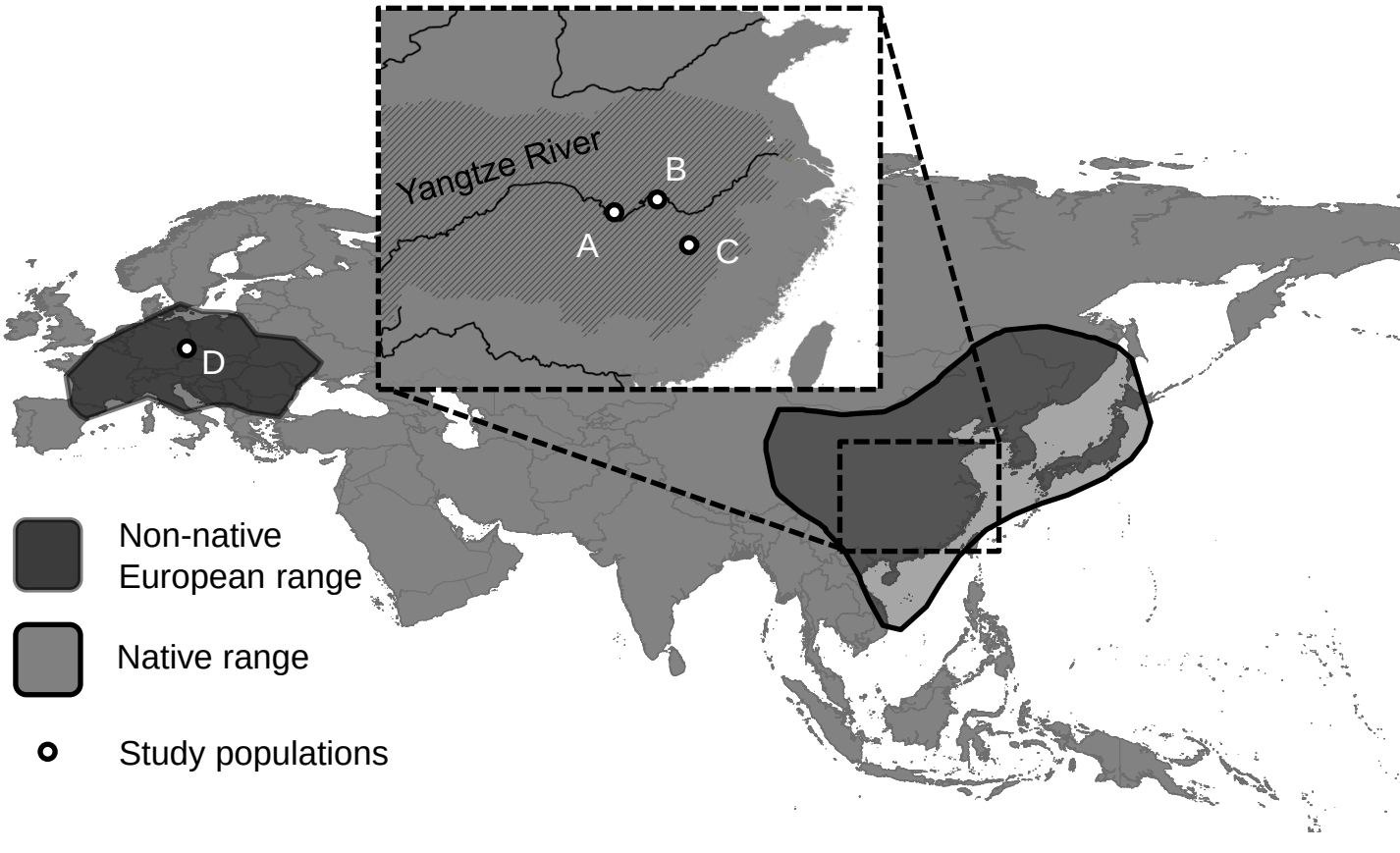
Schlegel, 1846)							
Perca fluviatilis Linnaeus, 1758	2	8.19, 4.62	68, 59	1	3	3.00	0.50
Gobio gobio (Linnaeus, 1758)	11	3.42 ± 2.19	55.9 ± 14.0	2	4	2.00	0.18
Cyprinus carpio Linnaeus, 1758	9	6.13 ± 1.25	52.9 ± 3.9	7	33	4.71	0.78
Carassius gibelio (Bloch, 1782)	12	4.57 ± 4.08	44.5 ± 13.8	8	34	4.25	0.67

FIGURE CAPTIONS

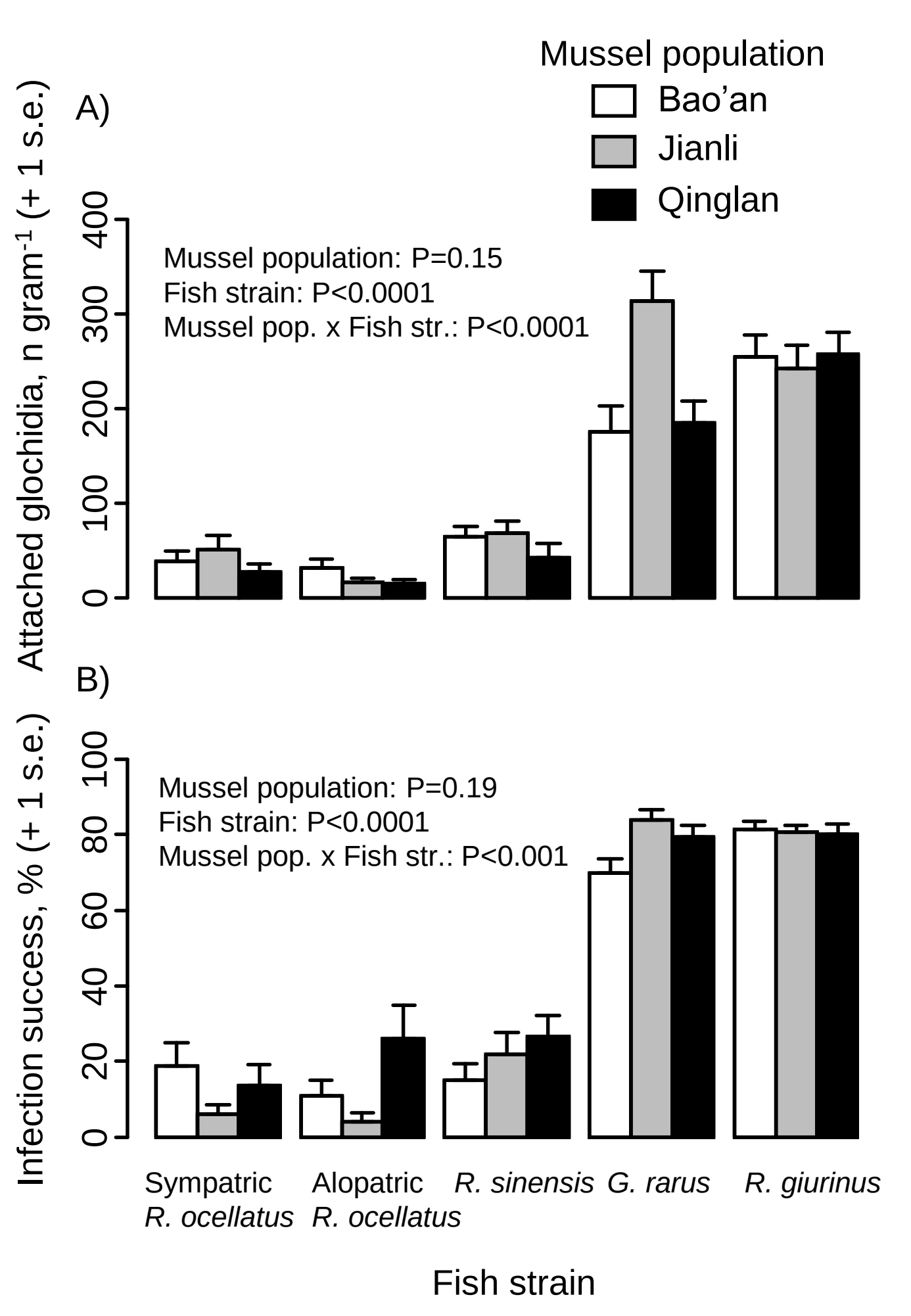
FIGURE 1 Study area with collection sites in the *Sinanodonta woodiana* native (A – Jianli, B - Lake Bao'an, C - Lake Qinglan; China) and non-native range (D – River Kyjovka; Czech Republic).

FIGURE 2 Metamorphosis success rate of *Sinanodonta woodiana* (population B - Lake Bao'an; China) on tested East Asian host fish species (median (dashed line), interquartile range (box), min/max (whiskers), and outliers > 1.5 x interquartile range displayed). Data from a previously tested non-native population (Douda, Vrtílek, Slavík, & Reichard, 2012) are also included for a comparison (grey boxes). Bitterling fishes (*R. sinensis*, *R. ocellatus* and *R. amarus*) are represented by hatched boxes. Boxes with different index letters (A, B, C) are statistically significantly different from each other (Tukey's HSD test, $p < 0.05$).

FIGURE 3 Population-level host compatibility of *Sinanodonta woodiana* with its native range hosts. The number of attached glochidia per gram of fish body weight (A) and infection success (B) versus *Mussel population* (populations A - Jianli, B - Bao'an, C - Qinglan) and *Fish strain* in the population-level experiment. Results of GLMM with the respective *p*-values for the effects of *Mussel population*, *Fish strain* and the interaction term are displayed (n = 14-16 per group).


Yangtze River
A
B
C
D
Non-native
European range
Native range
Study populations

NATIVE RANGE
NON-NATIVE RANGE
Metamorphosis success rate
0.0
0.2
0.4
0.6
0.8
A
A
A
AB
A
A
AB
C
C
B
ABC
Carassius auratus
Danio rerio
Rhinogobius giurinus
Hypoph. nobilis
Sinibotia superciliaris
Silurus meridionalis
Plagio. microlepis
Rhodeus sinensis
Rhodeus ocellatus
other hosts
Rhodeus amarus


Mussel population
Bao'an
Jianli
Qinglan
A)
Attached glochidia, n gram^{-1} (+ 1 s.e.)
0
100
200
300
400
Mussel population: P=0.15
Fish strain: P<0.0001
Mussel pop. x Fish str.: P<0.0001
B)
Infection success, % (+ 1 s.e.)
0
20
40
60
80
100
Mussel population: P=0.19
Fish strain: P<0.0001
Mussel pop. x Fish str.: P<0.001
Sympatric R. ocellatus
Alopatric R. ocellatus
R. sinensis
G. rarus
R. giurinus
Fish strain

Supporting Information Appendices

The role of local adaptation in shaping fish-mussel coevolution

Authors: Karel Douda, Huan-Zhang Liu, Dan Yu, Romain Rouchet, Fei Liu, Qiong-Ying Tang, Caroline Methling, Carl Smith, Martin Reichard

Table S1. List of fish species used as a comparative dataset (source: Douda, Vrtílek, Slavík and Reichard 2012, Biological Invasions, 14, 127-137) in the species-level host compatibility experiment (section 2.1).

Species name	Authority
Squalius (Leuciscus) cephalus	(Linnaeus, 1758)
Rutilus rutilus	(Linnaeus, 1758)
Gobio gobio	(Linnaeus, 1758)
Barbus barbus	(Linnaeus, 1758)
Cyprinus carpio	Linnaeus, 1758
Pseudorasbora parva	(Temminck & Schlegel, 1846)
Carassius gibelio	(Bloch, 1782)
Rhodeus amarus	(Bloch, 1782)

Table S2. Mean ± s.d. standard length (SL) and mean mean ± s.d. wet body mass (W) of experimental fish used in the population level host compatibility experiment (section 2.2).

Fish strain	SL (mm)	W (g)
syntopic *R. ocellatus*	33.7 ± 3.8	0.75 ± 0.29
allotopic *R. ocellatus*	31.0 ± 2.5	0.58 ± 0.14
R. sinensis	36.3 ± 3.9	0.97 ± 0.38
R. giurinus	39.5 ± 2.9	0.83 ± 0.18
G. rarus	34.8 ± 2.7	0.62 ± 0.18